Reference: *Biol. Bull.* **164**: 201–213. (April, 1983)

EGGS OF *PALAEMON MACRODACTYLUS*: II. ASSOCIATION WITH AQUATIC BACTERIA

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ABSTRACT

Eggs of the shrimp, *Palaemon macrodactylus,* were studied under various conditions to determine their susceptibility to colonization by aquatic bacteria. Under constant conditions in a laboratory system, bacterial populations monitored over the shrimp's two-week brooding period reached a maximum by seven days and stabilized. Nutrient addition increased bacterial populations substantially and retarded embryonic development, a probable result of hypoxia. Bacterial populations on eggs that had been detached from the egg mass and suspended in the system water increased by nearly an order of magnitude, as did populations on eggs brooded by females whose first pereiopods, or "cleaning chelipeds," had been excised. This result implied that preening activities of the first pereiopods were responsible for dramatically reducing bacterial populations. Bacterial populations on fertilized and unfertilized eggs responded alike to conditions of detachment and nutrient addition, indicating no apparent differences caused by mating, fertilization or development. Eggs extruded without benefit of attachment by the outer investment coat, a condition artificially produced by excising the pleopods, supported high bacterial populations. This may have resulted from additional nutrient escaping from the ooplasm or the greater susceptibility of the exposed vitelline envelope.

INTRODUCTION

Externally incubating crustacean embryos are exposed to the aquatic environment for extended durations. For example, the Pacific coast Dungeness crab, *Cancer magister,* broods its embryos for nearly 3 months, and the American lobster, *Homarus americanus,* has a 9 month incubation period. During this time, the embryos are a potential substrate for aquatic bacteria that are attracted to solid surfaces. Nutrient concentration at solid-liquid interfaces has been shown to attract bacteria (Marshall, 1976), and in low-nutrient (oligotrophic) environments, association with surfaces is frequently necessary for bacterial growth (Heukelekian and Heller, 1940; ZoBell, 1943). Several investigators (Henrici, 1933; Zobell and Allen, 1933; Marshall *et al.,* 1971; Corpe, 1973) have described the colonization and succession of microorganisms on surfaces of submerged glass slides. Within 5 days, complex microbial communities develop which include stalked and budding bacteria, diatoms, and protozoans.

In externally brooding decapods developing embryos are attached beneath the abdomen on the pleopods of the female. Water is circulated through the egg mass which acts like a filter by accumulating debris (Bauer, 1979). This build-up of organic debris and microorganisms is often detrimental to the host embryos. Fisher (1977) lists some reports of microbial epizoic diseases on decapods and suggests that microorganisms restrict gaseous exchange across the embryonic surface by both phys-

Received 20 September 1982; accepted 25 January 1983.

ical means and respiratory competition, causing asphyxiation of the embryo. Working with hatchery-incubating eggs of salmon and trout, Trust (1972) found that bacteria covering only 1–10% of the available surface of an egg could consume oxygen at a rate five times faster than the egg. Oppenheimer (1955) and Bell (1966) have suggested that enzymes and toxins produced by surface microorganisms could damage embryos. Bell and co-workers (1971) however, studied the bacterial flora of stream-incubating Pacific salmon eggs and suggested bacterial fouling occurs only after eggs die. They suggested that heavy fouling on live eggs occurs only in culture, where conditions stimulate bacterial growth.

Presumably, healthy decapod embryos exposed for long periods of time have some mechanism(s) to restrict the growth of microorganisms on their surfaces. Possibilities include "preening" by the female (Bauer, 1979), maternal secretions such as the antibacterial agglutinating exudate of the aquatic arthropod, *Limulus polyphemus* (Stagner and Redmond, 1975), and embryonic secretions, such as the antimicrobial glucanase (Horikoshi *et al.*, 1963), which is released by sea urchin eggs during the fertilization reaction (Epel *et al.*, 1969).

This study examines eggs of an estuarine shrimp, *Palaemon macrodactylus*, in an attempt to elucidate some of the factors that determine the number of bacteria that inhabit the surfaces. The term "eggs" is often used in a general sense to mean unfertilized eggs, fertilized eggs, or both. Where specific designation is relevant, the terms "unfertilized eggs" and "fertilized eggs" or "embryos" will be used.

Materials and Methods

The animals

Adult estuarine shrimp, *P. macrodactylus*, were collected from the mouth of the Petaluma River in northern California and transported to the University of California, Davis campus, where they were held in a closed recirculating system. They were placed in individual 8 × 15 cm compartments (10 cm water depth) in a 1.5 m × 30 cm plexiglass tank. A small jet of water was splashed into each compartment and was drawn through sand, pebbles, and oyster shell in an undergravel filter. Water temperature was maintained at 21–25°C; salinity fluctuated between 10–20 g/l; light:dark regime was 16:8 and animals were fed daily with live adult *Artemia salina* or frozen bay shrimp, *Crangon*. Mating of *P. macrodactylus* was most successful when a male was placed with a female a few days before her expected molt. If not mated, females extruded and attached unfertilized eggs, but they were usually removed or shed within 3 days.

Monitoring bacterial populations

Bacteria were enumerated by removing a cluster of attached eggs or embryos from the second pleopod of the brooding female and dissecting five connected eggs from the cluster as one sample. The samples were rinsed five times with sterile sea water diluted 1:2 (=1/3 SW) in a sterile porcelain dish, then homogenized in a sterile 5 ml Potter-Elvehjem tissue grinder (0.10–0.15 mm clearance). Using standard bacteriological procedures, the homogenized sample was diluted and plated in triplicate on a diluted concentration of Difco Marine Agar 2216 (=1/3 MA). Dilution was employed to simulate estuarine salinities; control plating showed that the dilution of nutrient in the media did not reduce bacterial counts. Bacterial colonies were counted after incubation for 3 days at 25°C. Egg clusters of 18 females were sampled

directly from the Petaluma River on 16 June 1981 to determine bacterial levels in nature.

Bacterial populations from embryos and unfertilized eggs attached to various females in the system were monitored intermittently over a 3-month period. In an eight-day span during this period, system water from individual compartments was simultaneously monitored with embryo samples to determine whether bacterial fluctuations within the system or over time would alter the trends observed on the embryo samples. To accomplish this, 15 water and embryo samples were collected from 4–6 compartments on three separate occasions within the 8 days. Water samples were plated and incubated as previously described for embryo samples.

Effects of attachment

Unattached, unfertilized eggs were obtained by excising the first or second pair of female pleopods immediately prior to egg extrusion. As the eggs were extruded, they fell unattached to the floor of the tank where they clumped together. Within hours, the eggs were rinsed and placed, as a clump, in moving, well-aerated system water, and bacterial populations were monitored. Clusters of about 50 detached embryos, that is, embryos artificially removed from the second pleopods of the female, were clipped from various females, rinsed three times in sterile 1/3 SW, and suspended in system water by a sewing thread wrapped around the connectants between the embryos. These detached embryos were suspended either in uninhabited compartments of the system or in 125 ml flasks with 100 ml of system water that was changed daily. Sterile aeration was provided via 1 ml pipettes inserted through vented stoppers in each flask. In five separate experiments, embryos were detached 7, 7, 10, 11, and 12 days after extrusion, and bacterial populations were monitored for 1–7 days after detachment. Embryos remaining attached to the female were also monitored for comparison.

The first or third pair of pereiopods, which correspond respectively to the "cleaning chelipeds" and "first walking legs" described by Bauer (1979), were excised from two mated females who had extruded eggs on the same day. The pereiopods were excised 3 days after oviposition, and bacterial populations were monitored intermittently thereafter until hatching. In two other females, the first pereiopods were excised 1 day and 6 days after their eggs were oviposited, and bacterial populations were monitored until hatching.

Effects of fertilization

Nonmated females attached extruding eggs for only a few days before removing them. To determine the effect of fertilization on the bacterial population, these eggs were monitored during this short attached period for comparison with fertilized eggs during the same period. In addition, unfertilized and fertilized eggs of the same age (1-day-old) were detached and suspended in flasks containing system water or system water plus nutrient (10% Difco Nutrient Broth) and bacteria were monitored 2 days later. This experiment was repeated once in 5% yeast extract and once using 1-day-old unfertilized eggs and 7-day-old fertilized eggs in system water and system water plus 10% yeast extract.

Effect of environmental nutrient and bacteria

Clusters of 5-day-old embryos were detached and suspended in four separate flasks of system water with 0%, 1%, 10%, and 20% concentrations of nutrient, a 1:2

dilution of Difco Marine Broth 2216 (=1/3 MB). System water and nutrient were replenished daily, and bacterial numbers were monitored for 7 days.

Attached and detached embryos were compared in their response to nutrient addition by suspending detached embryos (7 and 11 days old) in a 600 ml closed, aerated compartment with the parent female and her remaining complement of attached embryos. System water was supplemented with a 2% concentration of 1/3 MB and was replenished daily. Bacteria were monitored from attached and detached embryos.

Clusters of 7-day-old embryos were excised and suspended in separate aerated flasks of system water and system water plus inorganic nutrients (50 mg/l $NaNO_3$ and 50 mg/l NaH_2PO_4). Bacteria were monitored for the next 7 days from the two flasks of detached embryos and from those remaining attached on the female in a system compartment. Water in the flasks was not replenished, and nutrient was added only once.

A bacteria isolated from embryos of *P. macrodactylus* was incubated for 19 hours at 25°C in a 1/3 SW-yeast extract broth, centrifuged into a pellet, and re-suspended in sterile 1/3 SW at a concentration of 6×10^{10} cells/ml. Clusters of about 50 detached 1-day-old embryos were placed in test tubes containing 9 ml of this inoculum, dilutions of this inoculum, and sterile 1/3 SW. Embryo samples were taken at 2 hours and 24 hours to monitor bacterial populations.

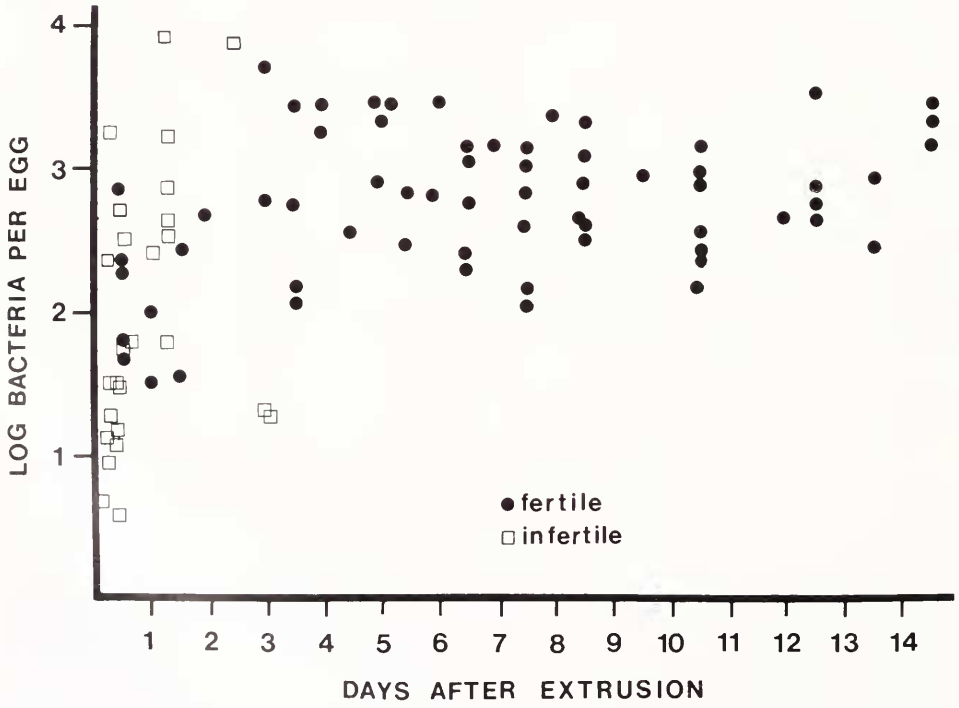


FIGURE 1. Numbers of bacteria on eggs attached to females held in the laboratory system. The period of attachment was 14 days for fertilized eggs and 1–3 days for unfertilized eggs. Each point on the graph represents the average count from a sample of five eggs detached from the second maternal pleopod, homogenized, then plated in triplicate on 1/3 MA.

TABLE I

Comparison of bacterial populations on embryos detached from the female pleopods with those remaining attached to the pleopods

Experiment	Age (days)	Number of bacteria/embryo	
		Attached	Detached
1.	$t_0 = 7$	1.5×10^2	
	8	4.6×10^2	7.1×10^2
	9	9.3×10^2	3.3×10^3
	10	8.1×10^2	4.0×10^3
2.	$t_0 = 7$	1.0×10^3	
	8	4.2×10^2	4.6×10^3
	10	1.4×10^3	7.2×10^3
	12	3.4×10^3	5.1×10^3
	14	2.1×10^3	6.8×10^3
3.	$t_0 = 10$	6.9×10^2	
	11	5.2×10^2	4.1×10^3
4.	$t_0 = 11$	8.3×10^2	
	12	1.4×10^3	3.9×10^3
	13	1.4×10^3	2.4×10^4
5.	$t_0 = 12$	8.7×10^2	
	13	1.1×10^3	9.8×10^3

t_0 = embryonic age at time of detachment.

RESULTS

The animals

Female *P. macrodactylus* held in the system molted every 16.3 (±3.5) days and eggs were extruded and attached to the pleopods after 76.5% of these molts, usually within 1 day. Fertilized eggs incubated an average 14.3 (±0.8) days, whereas unfertilized eggs remained attached only 1–3 days before being removed by the female.

Monitoring bacterial populations

Natural levels of bacteria determined from 18 females collected from nature averaged 118 bacteria/embryo (range = 10–600). Embryos attached to females held in the system showed an initial increase in bacterial numbers that leveled by the second week of brooding (Fig. 1). Samples monitored during the second week of brooding fluctuated near 10^3 bacteria/embryo. Populations in the system water over an 8-day period averaged 1.4 (±.64) $\times 10^5$, 3.2 (±.88) $\times 10^4$, and 1.6 (±.93) $\times 10^5$ bacteria/ml on three different sampling days. Plotting the ratios of bacteria per embryo to bacteria per milliliter system water showed no differences in the relative positions of each sample or the shape of the curve depicted in Figure 1. Unfertilized eggs showed increasing bacterial populations during the few days they were attached.

Effects of attachment

Unattached eggs (those extruded from females with excised first or second pleopods) had high bacterial populations of 1.7×10^5 bacteria/egg within 1 day and 2.7

TABLE II

Bacterial populations on embryos attached to females with excised first pereiopods, the "cleaning chelipeds"

Experiment	Age (days)	Number of bacteria/embryo
1.	$t_0 = 3$	2.8×10^2
	5	4.3×10^3
	7	3.3×10^3
	11	1.4×10^4
2.	$t_0 = 6$	5.5×10^3
	7	4.9×10^4
	10	1.5×10^4
	12	5.9×10^4
3.	$t_0 = 1$	7.4×10^1
	3	7.4×10^2
	5	4.9×10^3
	9	3.2×10^4
	10	7.1×10^4

t_0 = embryonic age at time of excision. Bacterial numbers for t_0 days were obtained prior to excision.

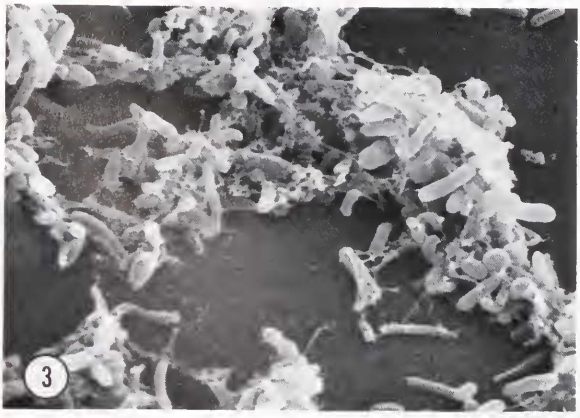
$\times 10^5$ bacteria/egg within 2 days after extrusion. During this time, the green yolk turned white, dissipated, and eventually only the translucent vitelline envelope remained. These unattached eggs did not have an outer investment coat (Fisher and Clark, 1983). Bacterial populations on detached embryos were higher than those remaining attached to the female pleopods (Table I). Numbers of bacteria on attached embryos were an average 72% less than those found on detached embryos, and those on embryos from the female with her walking legs excised were an average 73.8% less than those from the female with her cleaning chelipeds excised. Bacterial populations increased on all females with excised chelipeds (Table II), and scanning electron micrographs (Figs. 2–5) showed egg surfaces from normal females (with intact cleaning chelipeds) to have patchy distributions of bacteria.

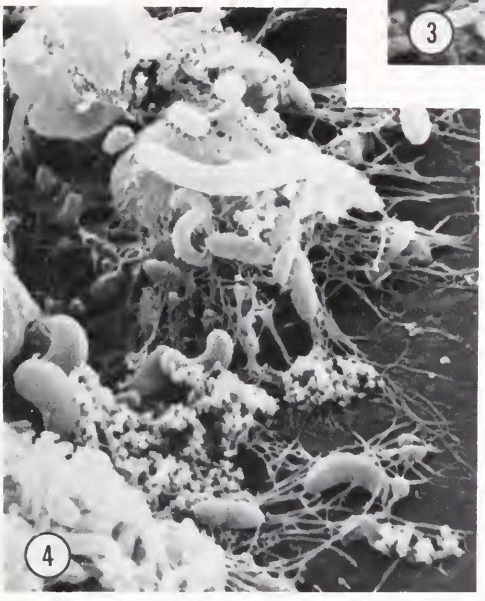
Effects of fertilization

Bacterial populations on attached eggs monitored over the first three days showed large variation and no distinguishing differences between fertilized and unfertilized eggs (Fig. 1). Eggs and embryos detached within 1 day of extrusion and suspended in system water or system water plus nutrient, had equivalent bacterial populations after 3 days (Table III). Likewise, populations on unfertilized eggs detached within 1 day responded in a manner nearly identical to populations of fertilized eggs detached after 7 days of brooding.

Effect of environmental nutrient and bacteria

Incremental concentrations of nutrient (1/3 MB) increased populations of bacteria on detached 5-day-old embryos (Fig. 6). Populations on control embryos (0%) increased one order of magnitude before leveling at 10^4 bacteria/embryo. Daily addition of 1% nutrient caused an initial increase of nearly two orders of magnitude that also stabilized. Bacteria on embryos held in 10% nutrient exceeded 6×10^5 bacteria/embryo in 2 days, but measurements were discontinued because of a fungal





FIGURES 2–5. Scanning electron micrographs of bacteria on the surfaces of embryos from normal females (cleaning chelipeds intact). In Figure 2 (80×), only a small portion of the embryo is inhabited by bacteria, but these bacteria appear capable of forming colonies as shown in Figure 3 (5,000×). The most heavily infested areas show a variety of microbial epizoots such as those shown in Figure 4 (7,500×). Patchiness may be due to preening or scraping by the female's cleaning appendages, which could account for the rolled mats of microorganisms shown in Figure 5 (240×).

infection. Bacterial populations on embryos suspended in 20% nutrient increased to 10^6 bacteria/embryo within 2 days and 10^8 bacteria/embryo within 7 days. Twelve-day-old embryos in 20% nutrient were approximately 2–3 days retarded in their development, as indicated by eyespot size and appearance, whereas embryos in the 0% and 1% nutrient developed within the normal time period.

Both attached and detached embryos monitored from the time of detachment, showed increased bacterial populations when held in a 2% concentration of 1/3 MB (Fig. 7), but bacterial counts on attached embryos were less (by an average 85.2%) than those on detached embryos. Hatching success and larval survival was poor from the detached embryos whereas hatching appeared to occur normally from the attached embryos and larval survival was excellent.

TABLE III

Comparison of bacterial populations on fertilized and unfertilized eggs detached from maternal pleopods and suspended in the same flask containing either system water or system water with a nutrient additive

Experiment	Unfertilized		Fertilized	
	Age (d)	Number of bacteria per egg	Age (d)	Number of bacteria per egg
1. No nutrient	1	1.7×10^3	1	7.2×10^1
	3	4.3×10^4	3	4.0×10^4
10% Nutrient Broth	1	1.7×10^3	1	7.2×10^1
	3	2.1×10^5	3	1.2×10^5
2. 5% Yeast Extract	1	5.1×10^2	1	6.5×10^2
	3	1.5×10^5	3	1.1×10^5
3. No nutrient	1	1.3×10^1	7	1.1×10^2
	2	3.2×10^3	8	5.6×10^3
	3	8.3×10^3	9	5.0×10^3
10% Yeast Extract	1	1.3×10^1	7	1.1×10^2
	2	3.2×10^4	8	1.9×10^4
	3	8.3×10^4	9	6.2×10^4

Seven-day-old embryos suspended in system water with a single addition of inorganic nutrients showed an increase in bacteria of two orders of magnitude after 1 day (Fig. 8); this number gradually declined during the experimental period and resulted in successful hatching of larvae. Embryos suspended in unreplenished system water without nutrient addition increased to 4.6×10^3 bacteria/embryo after 1 day then leveled until hatching. Those embryos remaining attached to the female maintained an average population of 10^3 bacteria/embryo throughout the experiment.

One-day-old embryos held in water of various concentrations of bacteria showed an early response to the higher concentrations, but such differences diminished after only 24 hours (Table IV). The highest counts on the embryos, 7.4×10^5 bacteria/embryo, were observed after 2 hours exposure to the highest inoculum (6×10^{10} bacteria/ml). By 24 hours, the bacterial count had decreased to 3.9×10^4 bacteria/embryo, while the bacterial count from the control tube increased from 6.3×10^2 (2 hours) to 6.0×10^3 bacteria/embryo by 24 hours. Bacterial numbers from this experiment cannot be compared with numbers from other experiments in this study since experimental conditions were different.

DISCUSSION

Bacterial populations on brooding embryos of the estuarine shrimp, *P. macrodactylus,* were restricted to approximately 10^3 bacteria/embryo during the major portion of the shrimp's 14-day incubation period in laboratory conditions. Increasing environmental nutrient increased the bacterial population (Fig. 6), and continuous addition of high levels of nutrient retarded the development of embryos. Respiration of bacteria and their physical presence on an embryo surface reduces the amount of oxygen reaching the embryo and, if sufficient numbers of bacteria are present,

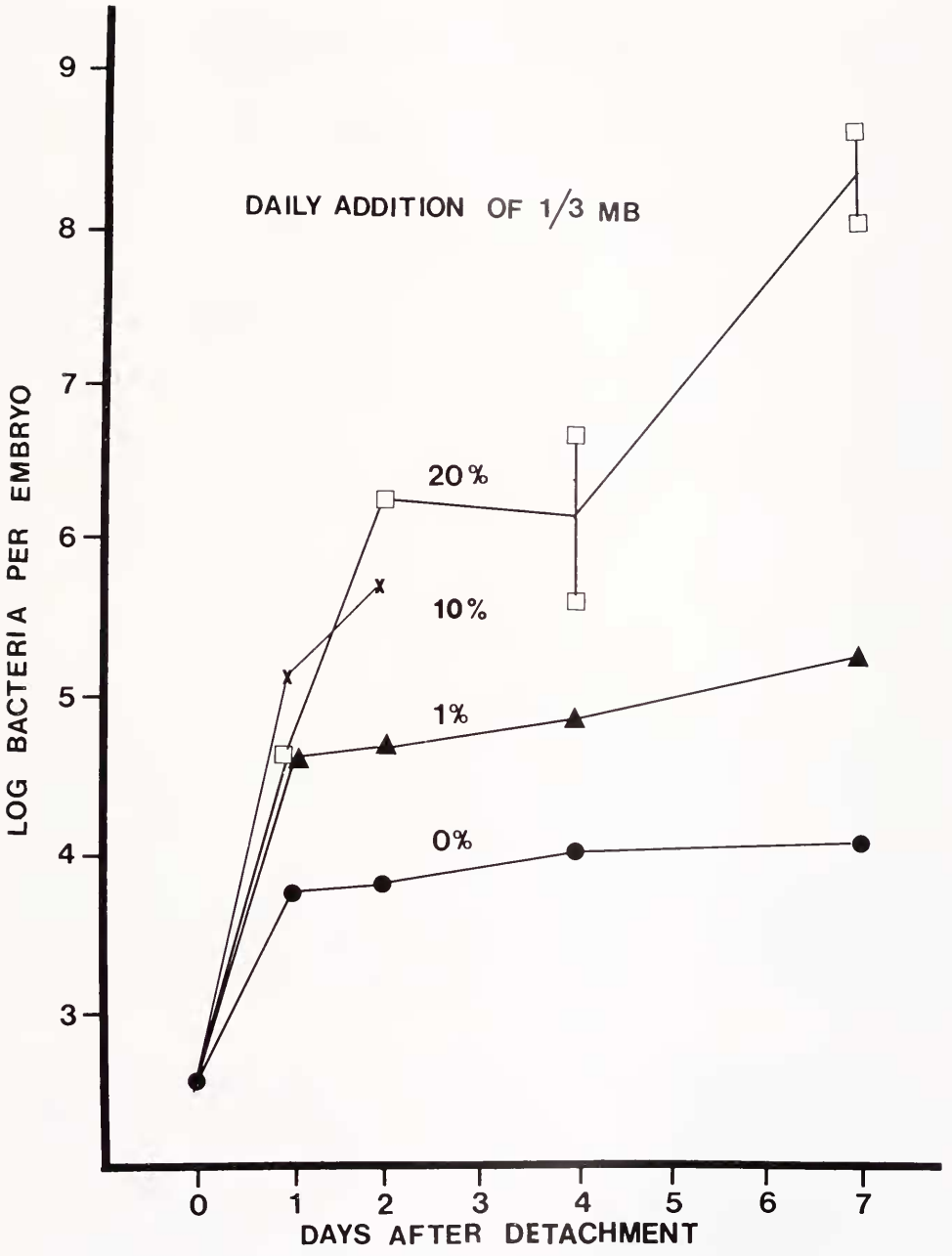


FIGURE 6. Numbers of bacteria on five-day-old embryos detached from a single female and suspended in different concentrations of nutrient (1/3 MB) that was added daily with the water change. Embryos held in 10% nutrient were infected by fungus two days after detachment and eventually died. Those held in 20% nutrient were 2–3 days retarded in development by the seventh day after detachment.

may create a respiratory stress condition for the embryo. Oxygen requirements of *P. macrodactylus* embryos increase dramatically at about 9 days (unpublished data) which may be a particularly vulnerable period.

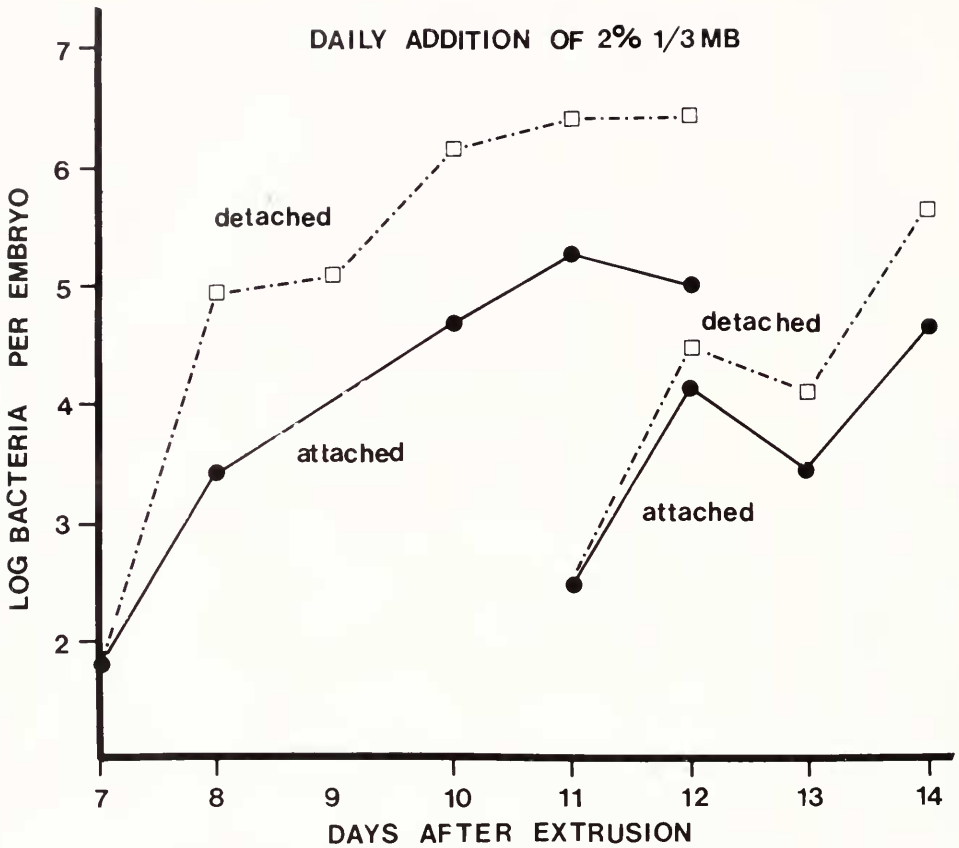


FIGURE 7. Numbers of bacteria on embryos detached from two females, one after seven days of brooding and the other after eleven days of brooding, and numbers of bacteria on embryos remaining attached to those females. Detached embryos were suspended in the same compartments with the respective donor female and the remaining complement of attached embryos. Nutrient (1/3 MB) was added at a 2% concentration with the daily water change. Hatching of the older embryos was successful for those remaining attached on the female, but detached embryos had poor hatching success and poor larval survival.

Another detrimental condition may result from bacteria inhabiting embryo surfaces. Bacteria isolated from the surfaces of *P. macrodactylus* embryos (Fisher, 1983) produced extracellular enzymes capable of degrading (among other substrates) chitin and lipid, two structural components of decapod cuticles and egg coats (Yonge, 1937). Bell (1966) suggested that bacterial release of degrading enzymes caused damage to fish egg coats and ultimately to the embryo. Such a phenomenon might also be expected in the decapods, since it has been well established that chitinolytic bacteria (Hess, 1937; Rosen, 1967) and possibly lipolytic bacteria (Baross *et al.*, 1978) are capable of penetrating the tough decapod exoskeleton to create shell diseases.

Eggs that were extruded without benefit of attachment (obtained by excising the first or second maternal pleopods before extrusion) lacked an outer investment coat provided by the female pleopods (Fisher and Clark, 1983). These unattached eggs rapidly deteriorated and, within 2 days, only the vitelline envelope remained ap-

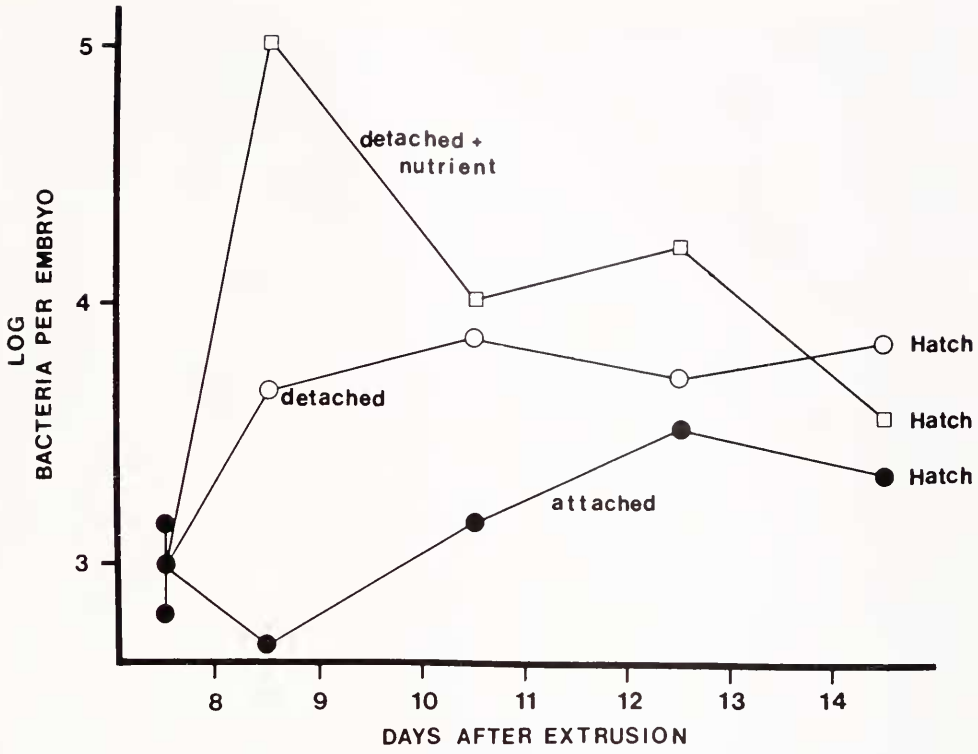


FIGURE 8. Numbers of bacteria on seven-day-old embryos detached from a single female and suspended in system water (detached) or system water plus a single addition of the inorganic nutrients $NaNO_3$ and NaH_2PO_4 at 50 mg/l each (detached + nutrient), and on embryos remaining attached to the female which were held in a system compartment (attached). Water was not replenished during the experiment. Successful hatching occurred from all treatments.

pearing as a translucent "ghost capsule." During this time, a high number of bacteria ($1.7–2.7 \times 10^5$ per egg) inhabited the surfaces, implying that a high and constant level of nutrient was available from the vitelline envelope itself or from leakage of ooplasm through the vitelline envelope.

TABLE IV

Bacterial populations found on one-day-old embryos detached from the same female and held in tubes of increasing concentrations of bacteria[1]

Initial inoculum (bacteria/ml)	Number of bacteria/embryo[2]	
	After 2 hours	After 24 hours
Control (=0)	6.3×10^2	6.0×10^3
6.0×10^4	1.1×10^3	7.8×10^4
6.0×10^6	1.2×10^3	1.1×10^4
6.0×10^8	7.9×10^3	4.2×10^4
6.0×10^{10}	7.3×10^5	3.9×10^4

[1] Bacteria for inocula were isolated from an embryo surface and cultured from a single colony.
[2] Numbers of bacteria were monitored from the same tubes held at room temperature 2 hours and 24 hours after inoculation.

Embryos that were detached from the brooding pleopods consistently had more bacteria than embryos remaining attached. A comparably high number of bacteria were found on embryos of females whose first pereiopods (cleaning chelipeds) had been excised, indicating that preening behavior may be responsible for controlling a major portion of the bacterial population. Scanning electron micrographs (Figs. 2–5) support this hypothesis by showing patchy distributions of bacteria, possibly corresponding to areas raked by cleaning setae of the first pereipods (Bauer, 1979). Infection of eggs by a fungus, *Lagenidium callinectes*, has also been shown to increase when the cleaning chelipeds are excised (Fisher, 1983).

Fertilization had no apparent effect on the bacterial numbers, since populations monitored on unfertilized eggs were the same as those on fertilized eggs under conditions of attachment, detachment, and nutrient addition. The initial number of bacteria in the environment was a relevant factor for only one day, after which populations on embryos held in high bacterial concentrations receded to levels found on embryos held in low bacterial concentrations. Similarly, populations on embryos treated with only a single addition of nutrient increased temporarily but eventually declined to levels found on untreated embryos.

Figure 8 depicts a single experiment that aids in summarizing some of the results of this study. Bacterial populations on detached embryos fluctuated around 10^4 bacteria/embryo while the populations on attached embryos were reduced by female preening activities to about 10^3 bacteria/embryo. By analogy, it can be estimated that nutrient in the Petaluma River (where natural samples were obtained) was capable of supporting 10^3 bacteria/embryo and was reduced by female preening to the measured 10^2 bacteria/embryo. A single addition of inorganic nutrient to the test water of detached embryos allowed the bacterial population to increase initially (Fig. 8), but numbers subsequently dropped to near the control level. This probably reflects competition between bacteria for space or nutrient. When nutrient is continuously added, bacterial populations are maintained at increased levels (Fig. 6). In this context, it is also interesting that high bacterial populations in water without additional nutrient did not remain associated with the embryos (Table IV). Further study may reveal a relationship between nutrient levels and bacterial attachment to embryos.

It has been shown here that *P. macrodactylus* embryos depend largely on low nutrient levels and maternal preening to restrict surface bacterial populations. The effect of nutrient on microbial populations inhabiting embryo surfaces has also been shown for the Dungeness crab, *Cancer magister* (Fisher, 1976). Since female crabs do not have the same preening ability as *P. macrodactylus* and carry roughly a million eggs, it is interesting to speculate whether crabs must depend on low nutrient levels or if there is some other form of defense.

Acknowledgments

Thanks to Wallis H. Clark, Jr. and Jack Meeks for their participation and cooperation in this study and Ann McGuire for her constructive criticism. Work was supported by a Sea Grant Traineeship and Sea Grant #NOAA 04-m01-189. I wish to dedicate this study to my teacher and friend, Edgar Herland Nilson.

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